



# Oxidative Stress Is Associated with an Increased Antioxidant Defense in Elderly Subjects: A Multilevel Approach

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## Abstract

**Background:** Studies of associations between plasma GSH-Px activity and cardiovascular risk factors have been done in humans, and contradictory results have been reported. The aim of our study was to assess the association between the scavenger antioxidant enzyme glutathione peroxidase (GSH-Px) activity in plasma and the presence of novel and classical cardiovascular risk factors in elderly patients.

**Methods:** We performed a cross-sectional study with baseline data from a subsample of the PREDIMED (PREvención con Dieta MEDiterránea) study in Spain. Participants were 1,060 asymptomatic subjects at high risk for cardiovascular disease (CVD), aged 55 to 80, selected from 8 primary health care centers (PHCCs). We assessed classical CVD risk factors, plasma oxidized low-density lipoproteins (ox-LDL), and glutathione peroxidase (GSH-Px) using multilevel statistical procedures.

**Results:** Mean GSH-Px value was 612 U/L (SE: 12 U/L), with variation between PHCCs ranging from 549 to 674 U/L (Variance = 1013.5;  $P < 0.001$ ). Between-participants variability within a PHCC accounted for 89% of the total variation. Both glucose and oxidized LDL were positively associated with GSH-Px activity after adjustment for possible confounder variables ( $P = 0.03$  and  $P = 0.01$ , respectively).

**Conclusion:** In a population at high cardiovascular risk, a positive linear association was observed between plasma GSH-Px activity and both glucose and ox-LDL levels. The high GSH-Px activity observed when an oxidative stress situation occurred, such as hyperglycemia and lipid oxidative damage, could be interpreted as a healthy defensive response against oxidative injury in our cardiovascular risk population.

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## Introduction

Coronary heart disease (CHD) is a major cause of morbidity and mortality in the developed world [1]. Atherosclerosis, characterized by the accumulation of cholesterol deposits in large and medium-sized arteries, is the most common pathologic process underlying cardiovascular disease and is often clinically manifested as coronary, cerebrovascular, and/or peripheral arterial disease [2]. An imbalance between antioxidant and oxidant-generating system that leads to oxidative stress has been proposed in the pathogenesis of atherosclerosis [2]. In particular, the oxidation of low density lipoproteins (LDL) by free radicals plays a central role in the formation, progression, and rupture of atherosclerotic plaques [2].

Mammalian cells are, however, protected from free radicals by a wide range of antioxidants such as the scavenger antioxidant enzymes [3]. Glutathione peroxidase is the general name for a family of multiple isozymes that catalyze the reduction of  $H_2O_2$  or organic hydroperoxides to water or the corresponding alcohols using reduced glutathione (GSH) as an electron donor. In mammals, 8 glutathione peroxidases (GPx1–GPx8) have been identified to date, including both selenium-containing GPxs (GPx1–4 and 6) and their non-selenium congeners (GPx5, 7 and 8) [4].

Oxidative stress elicits an induction of antioxidant enzymes, as reported in a recent systematic review [5]. However, most studies were done in animal models; studies that have analyzed this association in humans have reported conflicting results [6–15].

The aim of the present study was to assess the association between the scavenger antioxidant enzyme glutathione peroxidase (GSH-Px) activity in plasma and novel and classical cardiovascular risk factors in elderly individuals at high risk for cardiovascular disease.

## Methods

### Study design

A cross-sectional study with baseline data from a subsample of the PREDIMED (PREvención con Dieta MEDiterránea) study was performed. The PREDIMED study is a large, parallel-group, multicenter, randomized, controlled, clinical trial aimed at assessing the effects of the traditional Mediterranean diet (TMD) on the primary prevention of cardiovascular disease ([www.predimed.es](http://www.predimed.es) and [www.predimed.org](http://www.predimed.org)). The PREDIMED detailed protocol of the study has been previously published [16].

### Subjects

Of the 7,447 participants aged 55 to 80 years from 8 Spanish PHCCs who were randomized to the PREDIMED study groups, 1,069 were randomly selected a posteriori for plasma measurements of glutathione peroxidase-1 activity and included in the present study. Inclusion criteria were the presence of diabetes or at least 3 CHD risk factors: current smoking; hypertension (systolic blood pressure  $\geq 140$  mm/Hg, diastolic blood pressure  $\geq 90$  mm/Hg, or treatment with antihypertensive drugs); dyslipidemia (high-density lipoprotein [HDL] cholesterol  $< 40$  mg/dL for men and  $< 50$  mg/dL for women, LDL cholesterol  $> 160$  mg/dL, or treatment with cholesterol-lowering drugs); overweight or obesity (body mass index (BMI)  $> 25$  kg/m<sup>2</sup>), or family history of premature CHD. Exclusion criteria were history of cardiovascular disease, any severe chronic illness, drug or alcohol addiction, history of allergy or intolerance to olive oil or nuts, or low predicted likelihood of changing dietary habits according to the

stages of change model. Individual eligibility was based on a screening visit by the primary care physician.

### Baseline assessments

The baseline examination included the administration of 3 types of questionnaire: 1) a validated food frequency questionnaire [17] and an assessment of the degree of adherence to the TMD, assigning a value of 0 or 1 to each of 14 questionnaire items [18], with energy and nutrient intake calculated from Spanish food composition tables [19]; 2) the Minnesota Leisure Time Physical Activity Questionnaire, which has been validated for its use in Spanish men and women [20,21]; and 3) a 47-item general questionnaire assessing life-style, health conditions, smoking habits, sociodemographic variables, history of illness, and medication use. Weight and height were measured with calibrated scales and a wall-mounted stadiometer, respectively. Waist circumference was measured midway between the lowest rib and the iliac crest using an anthropometric tape. Trained personnel measured blood pressure in triplicate with a validated semi-automatic sphygmomanometer (Omron HEM-705CP, The Netherlands) with the patient in a seated position after a 5-minute rest.

### Laboratory analysis

Biological samples were obtained after an overnight fast, coded, shipped to central laboratories, and frozen at  $-80^\circ\text{C}$  until the assay. Plasma glucose and lipid analyses were performed in a PENTRA-400 autoanalyzer (ABX-Horiba Diagnostics, Montpellier, France). Soluble HDL cholesterol was measured by an accelerator selective detergent method (ABX-Horiba Diagnostics, Montpellier, France) and LDL cholesterol was calculated by the Friedewald equation whenever triglycerides were  $< 3.4$  mmol/L. Quality control was performed with UNITY External Quality Assessment (BIO-RAD, Hercules, CA, USA). Circulating oxidized LDL (ox-LDL) plasma levels were measured by a commercial enzyme-linked immunoabsorbent assay (Mercodia AB, Uppsala, Sweden). Intra- and inter-assay coefficients of variation were 2.8% and 7.3%, respectively. Plasma GSH-Px activity (GSH-Px; EC 1.11.1.9) was measured by a Paglia and Valentine [22] modification method using cumene hydroperoxide (Ransel RS 505, Randox Laboratories, Crumlin, UK) as a glutathione oxidant. Intra- and inter-run imprecision were 3.6% and 5.43%, respectively.

### Statistical analyses

Participants were divided into quintiles of plasma GSH-Px concentration based on the sample distribution. We ensured that the result was not due to multiple comparisons, so we conducted the Holm adjustment [23,24]. This method is just as simple and generally applicable as the Bonferroni method, but much more powerful [25,26].

We applied multilevel statistical procedures [27] to investigate both the association between individual cardiovascular risk factors and GSH-Px and to what extent differences between PHCTs may account for any variation in the outcomes. We modeled individuals (level-1 units) as nested within 8 PHCTs (level-2 units). We modeled the continuous outcome (GSH-Px) using the multilevel linear regression to allow for within-center correlation, applying the Full Maximum likelihood method of estimation. Initially, we examined whether there was a variation between Spanish PHCTs in GSH-Px activity by fitting an unconditional model with no predictors at any level, only the intercept and random errors at the individual and PHCT levels. The second model estimated the effect of individual (level 1) covariates on the outcome and whether these effects varied by PHCT (i.e., we

allowed for level-2 random effects). We confirmed the appropriateness of modeling continuous variables as linear (fractional polynomials method).

All statistical tests were 2-sided at the 5% significance level. Analyses were carried out using the HLM for Windows multilevel package, version 10.1, Stata/SE version 9.1 (Stata Corp.).

### Research ethics

The study followed the principals contained in the Helsinki Declaration and successive revisions and the standards of good clinical practice. The protocol was approved by the Committee on Clinical Research Ethics (CEIC) of the Institut d'Investigació en Atenció Primària (IDIAP) Jordi Gol, and participants signed an informed consent. Data confidentiality was guaranteed according to the pertinent laws of Spain (Ley Orgánica de Protección de Datos de Carácter Personal, 15/1999, December 1).

### Results

Of the 1,060 included participants, 577 (54.4%) were female. The mean age was 66.7 (SD: 8.0) years. GSH-Px activity showed a normal distribution. Plasma glucose and ox-LDL levels increased across GSH-Px quintiles ( $P < 0.05$ ). (Table 1).

Table 2 shows the results of both the unconditional and the adjusted model for the association between GSH-Px and cardiovascular risk factors. From the results of the unconditional model, the GSH-Px mean value was 612 U/L (SE: 12 U/L), with a significant variation between PHCCs, ranging from 549 to 674 U/L (Variance = 1013.5;  $P < 0.001$ ). The variation in the mean level of GSH-Px was 15 times greater within PHCCs than between PHCCs (Variance = 15266.6;  $P < 0.001$ ). Thus, 93.8% of the variability in GSH-Px activity was between participants within centers rather than between PHCCs. The adjusted model (Table 2) showed a reduction in the mean GSH-Px value to 577 U/L (SE: 50 U/L), with a range of variation between PHCCs from 499 to 656 U/L. Results of this model showed that glucose and oxidized LDL were significantly positively associated with GSH-Px activity ( $P < 0.05$ ).

### Discussion

This study assessed the association between GSH-Px activity and classical and novel cardiovascular risk factors in an elderly population with high cardiovascular risk. We identified a positive association between plasma GSH-Px activity and glucose and ox-LDL levels. The associations were moderately strong and linear,

**Table 1.** Participant characteristics by quintiles of serum glutathione peroxidase activity (U/L).

	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P value for trend*
	<507	507–567	567–618	618–685	>685	
	(n = 213)	(n = 219)	(n = 212)	(n = 215)	(n = 210)	
Age (years)	67.1 ± 9.2	66.2 ± 8.8	67.5 ± 7.9	66.6 ± 6.7	66.3 ± 7.1	1.000
Sex (female), n (%)	122 (57.1)	116 (52.5)	123 (58.6)	113 (53.3)	107 (50.7)	1.000
Current smokers, n (%)	46 (21.8)	48 (22.4)	46 (22.1)	37 (17.5)	30 (14.5)	1.000
Diabetes, n (%)	94 (44.3)	99 (45.6)	96 (45.7)	112 (52.8)	118 (56.5)	0.076
Hypertension, n (%)	170 (79.7)	177 (81.1)	172 (82.2)	180 (84.8)	160 (76.8)	1.000
Systolic blood pressure (mmHg)	156 ± 61	159 ± 61	153 ± 20	158 ± 54	157 ± 62	1.000
Diastolic blood pressure (mmHg)	88 ± 63	89 ± 63	84 ± 11	89 ± 56	89 ± 64	1.000
Medication use, n (%)						
Antihypertensive agents	153 (73.2)	154 (74.0)	151 (73.7)	156 (75.7)	139 (67.8)	1.000
Lipid-lowering agents	78 (37.3)	96 (46.2)	89 (43.4)	84 (40.8)	95 (46.6)	1.000
Insulin	13 (6.2)	14 (6.8)	7 (3.4)	21 (10.3)	17 (8.3)	1.000
Oral hypoglycemic agents	57 (27.1)	55 (26.4)	55 (26.8)	68 (33.2)	71 (34.6)	0.435
Aspirin or other antiplatelet agents	52 (24.8)	40 (19.1)	43 (20.7)	63 (30.4)	49 (19.8)	1.000
Waist circumference (cm)	107.6 ± 88.8	104.3 ± 62.9	101.6 ± 53.7	97.8 ± 10.3	98.3 ± 11.1	0.468
EEPA leisure time (kcal/day)	224 ± 181	237 ± 195	277 ± 250	273 ± 253	267 ± 240	0.216
Glucose (mg/dL)	117 ± 34	118 ± 35	119 ± 34	125 ± 40	126 ± 39	0.042
Cholesterol (mg/dL)						
Total	203 ± 30	205.8 ± 32.3	209.2 ± 31.2	210.8 ± 35.6	207.5 ± 35.0	0.480
High density lipoprotein (HDL)	50.4 ± 10.5	50.7 ± 10.0	53.2 ± 10.0	52.6 ± 10.7	52.3 ± 10.2	0.288
Low density lipoprotein (LDL)	126 ± 26	128 ± 27	131 ± 26	133 ± 28	130 ± 29	0.216
LDL/HDL cholesterol ratio	2.52 ± 0.62	2.56 ± 0.59	2.51 ± 0.58	2.59 ± 0.61	2.52 ± 0.58	1.000
Triglycerides (mg/dL)	132 ± 63	133 ± 62	124 ± 53	124 ± 55	122 ± 65	0.435
Oxidized LDL (U/L)	70 ± 24	74 ± 26	74 ± 26	76 ± 26	78 ± 27	0.042

Abbreviations: EEPA, daily energy expenditure in leisure-time physical activity. Data are shown as mean ± SD, median (interquartile range), or percentage.

\* P value for trend adjusted for multiple testing using Holm correction [24]. One-factor analysis of variance, nonparametric Kruskal Wallis test, and chi-square test were used as appropriate.

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**Table 2.** Fixed and random parameters from a multilevel linear regression model of GSH-Px activity.

	Adjusted Beta coefficient <sup>a</sup>	Standard Error	P value
<b>UNCONDITIONAL MODEL</b>			
Fixed parameters			
Intercept	612	12.43	<0.001
Random parameters <sup>b</sup>			
PHCC-level Variance	1013.51		<0.001
Individual-level Variance	15266.61		
<b>ADJUSTED MODEL</b>			
Fixed parameters			
Intercept	577	50.07	<0.001
Glucose (mg/dl)	0.31	0.13	0.021
Sex (female vs. male)	−9.27	10.34	0.370
Age (years)	−0.53	0.54	0.330
EEPA leisure time (kcal/day)	0.02	0.02	0.251
Smoking (current smoking vs. nonsmoker)	−19.50	11.88	0.101
Insulin (treatment vs. no treatment)	24.66	18.17	0.175
Oral hypoglycemic agents (use vs. no use)	10.36	11.58	0.371
Oxidized LDL (mg/dL)	0.47	0.20	0.017
LDL/HDL cholesterol	2.23	8.23	0.786
Waist circumference (cm)	−0.10	0.08	0.224
Random parameters <sup>b</sup>			
PHCC-level Variance	1600.17		<0.001
Individual-level Variance	14640.03		

Abbreviations: EEPA, daily energy expenditure in leisure-time physical activity. PHCC, primary health care center.

<sup>a</sup>Adjusted  $\beta$ , regression coefficient of the association between each variable in the model and GSH-Px activity, controlling for the other variables in the model.

<sup>b</sup>Random parameters are multilevel measures of outcome variation. PHCC was considered as random.

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and persisted after adjustment for age, sex, and other possible confounders.

Although several studies have reported an association between cardiovascular risk factors and GSH-Px, they included small sample sizes and report contradictory results [6–15]. Furthermore, the results shown were either unadjusted [7,8,10–13] or adjusted only by age or sex [6,9,14,15]. Whereas several studies examined the association between GSH-Px activity and diabetes and reported lower serum GSH-Px activity in patients with type 2 diabetes than in non-diabetic participants [6,7,14], other studies found increased GSH-Px activity in diabetic patients, compared to control group [9,15]. Finally, one study found no significant difference in GSH-Px activity between three study groups (diabetic patients with and without hypertension and pre-diabetic patients) [8]. In other results, two of these studies reported a positive relationship between BMI and the antioxidant activity of GSH-Px [9,12], another study found significantly lower erythrocyte GSH-Px activity in obese women compared to normal weight women [10], and one study found no significant differences in GSH-Px activity between patients with essential hypertension and age- and sex-matched healthy controls older than 65 years [13].

The biological oxidative effects of reactive oxygen species (ROS) on lipids, DNA, and proteins are controlled by a wide spectrum of exogenous antioxidant mechanisms, such as vitamins and phenolic compounds in diet, and also by endogenous antioxidants such as the scavenger enzymes, among them GSH-Px [28]. Hyperglycemia is a situation in which ROS are generated [29]. In turn, ROS production induces GSH-Px generation at DNA transcriptional

level [30]. Thus, high GSH-Px activity may result from a preservation of the enzyme by a high antioxidant status (with low generation of ROS) or from increased GSH-Px production stimulated by ROS. Therefore, GSH-Px activity may serve as an indicator of the balance between oxidative status and the bioscavenging of ROS by antioxidants. In the present study we measured the *in vivo* ox-LDL as a marker of oxidative stress in order to examine the relationship between oxidative stress or damage and GSH-Px antioxidant enzyme activity. In our cardiovascular risk population, mean ( $\pm$  standard deviation) ox-LDL values ( $74 \pm 26$  U/L) were higher than those obtained in a healthy population ( $49 \pm 22$  U/L) using the same method and antibodies as in our study [31]. The positive linear relationship obtained between GSH-Px and both glucose and ox-LDL in our population would be compatible with enhanced production of GSH-Px when oxidative status is increased.

Induction of GSH-Px activity has been proposed as the mechanism by which preconditioning exerts protection in myocardial infarction [30]. In addition, overexpression of intracellular GSH-Px in transgenic animal models has shown to prevent postischemic free radical injury [32]. Cardiovascular risk factors present in our population, such as hypertension, diabetes, hyperlipidemia, and obesity, have been previously linked to oxidative stress and oxidative damage [33,34]. In this population, the fact that a high GSH-Px activity is observed when an oxidative stress situation occurs, such as hyperglycemia and lipid oxidative damage, could be interpreted as a healthy defensive response against the oxidative injury.

In our study, 93.8% of the in the variability GSH-Px activity was due to variability among participants within PHCCs rather than differences between PHCCs. After accounting for individual characteristics, the individual variability was reduced to 90.1%. It can hardly be expected that factors such as presence of genetic variants or selenium levels (a GSH-Px cofactor) influence GSH-Px activity [35]. In selenium-deficient patients, selenium supplementation increases enzymatic antioxidant activity such as GSH-Px and decreases lipid peroxidation [36]. The major sources of selenium are plant foods, meat, and seafood, but the selenium content of foods varies geographically depending on soil and water concentrations and the use of selenium-containing fertilizers [37]. Selenium intake in southern Spain has been reported to be above the Recommended Dietary Allowance (RDA) [38]. However, it is unknown whether selenium intake varies among Spanish populations and whether this could explain part of the variability found within PHCCs in our Spanish population.

The potential of selenoproteins such as selenium-containing GPxs (GPx1–4 and 6) to protect against oxidative stress led to the expectation that selenium would also be protective against type 2 diabetes and other cardiovascular risk factors. However, more recent findings from observational studies and randomized clinical trials have raised concerns that high selenium exposure may lead to type 2 diabetes, insulin resistance, or hyperlipidemia [39]. Additional evidence is needed to provide new insights into the role of selenium and of specific selenoproteins in human biology, especially to clarify the underlying mechanisms linking selenium to chronic disease endpoints. Further epidemiological studies and randomized clinical trials across populations with a different selenium status should be conducted to determine the causal effect of selenoproteins on the development of cardiovascular risk factors and diseases.

This study has strengths and limitations. A strength is its large sample size, geographically widespread within Spain. In addition, the study was carefully conducted using standardized protocols. The multilevel methodological approach allowed us to control for clustering within the PHCCs, thus assessing both the individual independent factors that may influence outcomes and whether there was variability in the plasma GSH-Px activity across PHCCs.

One of the limitations of our study is its cross-sectional design, which can identify associations but not causality. Moreover, cross-sectional studies are notably subject to confounding; for this reason, the analysis was adjusted for possible confounding factors. A large cross-sectional study such as this one contributes to the establishment of new hypotheses for large prospective studies and clinical trials. The relationship between selenoproteins such as GSH-Px and cardiovascular risk factors is undoubtedly complex. Future studies should genotype participants and investigate the potential interactions between genotype, selenium intake or status, and selenoproteins. Besides, they should explore whether other individual factors or contextual features (such as factors linked to

geographical or environmental characteristics of PHCTs) may account for variation in the GSH-Px values.

Our study focused on an elderly population and cannot be extrapolated to a general population. Another potential limitation is that a survival bias could have underestimated the association between GSH-Px activity and cardiovascular risk factors. It is possible that subjects with diabetes, obesity, or hypertension and low GSH-Px activity may have died or developed a cardiovascular disease, and therefore could not be included in our study. Moreover, we could not take into account the duration and control of diabetes in our analyses because these data were not available.

In conclusion, a positive linear association was observed between plasma GSH-Px activity and glucose and ox-LDL levels in an elderly population with high cardiovascular risk. The high GSH-Px activity observed when an oxidative stress situation occurs, such as hyperglycemia and lipid oxidative damage, would be compatible with an increase in antioxidant defenses against oxidative injury in our cardiovascular risk population. Further epidemiological studies and randomized clinical trials are needed to assess the impact of selenoproteins on the development of cardiovascular risk factors and diseases and the cause-effect relationships between hyperglycemia, oxidative status, and GSH-Px in elderly populations at high cardiovascular risk.

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Conceived and designed the experiments: MAM-G JS-S RE DC MF FA EG-G JL VR-G GTS MIC. Analyzed the data: GFM TR-B IS. Wrote the paper: GFM MFitó RE JBG MB MIC. Approved the final version of the article to be published: All authors.

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